Table of Contents

LARGE-SCALE Cell preparation of bacterial genomic DNA

1. Grow 100 ml culture of bacterial strain to saturation.
2. Pellet cells for 10 min at 6000 rpm (use GSA rotor)
3. Resuspend cells gently in 9.5 ml TE buffer. Add 0.5 ml of 10% SDS a
50 ul of 20 mg/ml proteinase K. Mix thoroughly and incub
1 hr at 37°C.
4. Add 1.8 ml of 5M NaCl and mix thoroughly.
5. Add 1.5 ml CTAB/NaCl solution. Mix thoroughly and incu
20 min at 65°C. (can stay for a moment)
6. Add an equal volume (13 ml) of chloroform/isoamyl alc
Extract thoroughly. Spin 10 min at 7000 rpm (use SS34
room temperature to separate phases.
7. Transfer aqueous supernatant to a fresh tube using a
wide-bored pipet.
8. Add an phenol/chloroform/isoamyl alcohol. Extration the
equal volume
Repeat three times.
9. Add an equal volume of chloroform/isoamyl alcohol.
10. Add 0.6 volume isopropanol and mix. (-20°C overnight)
(-20°C can help to precipitate.)

Notebook Number: _____

Date: 07/28/99

Continue:

11. Transfer the precipitate to 1ml of 70% ethanol in a fresh tube (just brown-red one), by hooking it on the end of a Pasteur pipet that has been bent (by heating it) and sealed.

12. Drying the pellet (just in the air). Remove white and stringy DNA to the 4 2ml TE buffer.

13. Do the electrophoresis of nucleic acid (there are a lot of DNA and RNA)

14. Use Rnase to digest the genomic DNA overnight.

Genomic DNA (continue)

1. Use EcoR I to do restriction. put { genomic DNA 27 ul / buffer (H) 9 ul / EcoR I 4 ul } → Digest genomic DNA overnight.

2. do the electrophoresis at 60 V
put 109 ul digested DNA (90 ul digested DNA
19 ul dye)
the result is not good.

Extracting Plasmid DNA of E.coli (Qiagen Midi and Maxi Prot.)

1. Inoculate a 5-ml LB medium with E. coli 37°c shake to afternoon

2. Inoculate 200ml medium Grow at 37°c ~~for~~ overnight.

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Date: 07/30/99

Extracting plasmid DNA. (continue)

3. Harvest the bacterial cells by centrifugation at 6000g for 15min at 4°C

4. Resuspend the bacterial pellet in 4 ml of buffer P1.

5. Add 4ml of buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate at room temperature for 5 min.

6. Add 4 ml of chilled buffer P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 15 min.

7. Centrifuge at 20000 g for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.

8. Re-centrifuge the supernatant at ≥20,000 xg for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly. ~~Alternatively, the sample can be fill~~

9. Equilibrate a QIAGEN-tip 100 by applying 4ml Buffer QBT. and allow the column to empty by gravity flow.

10. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.

11. Wash the QIAGEN-tip with 10ml (two times) buffer QC.

12. Elute DNA with 5 ml buffer QF.

13. precipitate DNA by adding 3.5 ml room-temperature isopropanol to the eluted DNA. Mix and Centrifuge immediately at ~~15000~~ 13000 rpm g for 30min at 4°C. Carefully decant ~~the~~ the supernatant.

14. Wash DNA pellet with 2ml of room-temperature 70% ethanol, and centrifuge at ~~15000~~ 13000 rpm g for 10min. Carefully decant the supernatant without

disturbing the ~~pette~~ pellet.

15 Air-dry the pellet for 5-10 min. and redissolve the DNA in a suitable volume of buffer → (TE buffer → 1/2 ml ?)

16 Do electrophorisis to check it. → to digest. → next page

8/3/99

High-efficiency transformation by electroporation

1. Inoculate a single colony of E Coli cells into 5 ml LB medium. Grow 5 hr to overnight at 37°C with moderate shaking

2. Inoculate 2 ml of the culture into 500 ml LB medium in a sterile flask. Grow at 37°C ~~shaking at~~ in ~~incubator~~ shaker to an OD_{600} of ~~0.5~~ 100 125. (if > 0.7 need more time.)

3 Chill cells in an ice-water bath 10 to 15 min and transfer to a prechilled 50ml centrifuge bottle.

4. Centrifuge cells ~~20 min~~ 10 min at ~~4200~~ 8000 rpm. 2°C

5. Pour off supernatant and ~~respend~~ resuspend the pellet in ice-cold water. Add 25ml ice-cold water and mix well. Centrifuge cells as in step 4 at 10min 8000 rpm. repeat 2 times. (throw away the supernatant to the bleach bottle including)

6. Pour off supernatant immediately and resuspend the pellet by swirling in remaining liquid.

7. Add another ml ice-cold water, mix well, and centrifuge again in step 4.

8 Pour off supernatant immediately and resuspend the pellet by swirling in remainning liquid.

~~9~~7. Put remainning liquid to the eppendorf tube. centrifuge it at 12000rpm 5min. pour off by using vacuum

8. add 100 ul water to the tube. mix well. put it on ice.
(含乳管为管)

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4 6

Notebook Number:

Date: 08/03/99

x10 Set the electroporation apparatus to 2.5kV, 25uF Set the pulse controller to 200 or 400 ohms? (Under the machine instruction)

11 Add 1uL plasmid DNA (PGP 704) in to tubes containing fresh or thawed cells (on ice). Mix by tapping the tube or by swirling the cells with the pipettor and 20uL bacterial

12 Transfer the DNA and cells into a cuvette that has been chilled 5 min on ice. Shake slightly to settle the cells to the bottom, and wipe the ice and water from the cuvette with a Kimwipe between

13 Place the cuvette in to the sample chamber

14 Apply the pulse by pushing the button of flipping the switch

15 Remove the cuvette. ~~Immediately add~~ SOC/LB broth immediately and transfer it to a sterile culture tube with a pasteur pipet. Incubate ~~at 37°C~~ 60 min with moderate shaking at 37°C

16 Plate aliquots of the transformation culture on LB plates containing antibiotics. including LB broth 100uL

08/03/99

Plasmid digest

PGP ~~704~~	1uL
10x buffer	1uL
Dnasefree H2O	7.5uL
ECoR I	0.5uL

37°C 2 hr → 65°C for 15 min

run 5uL on gel

Dilution 10^{-6} 100uL to LB DAP

10^{-5} 10^{-2} 100uL to LB DAP DAP

result

10^{-5} 10^{-2} zero 2-3 v

10^{-6} 100 colonies

To electrophoresis.

1. 15 ul marker (1kb) first line
2. 65 ul digested DNA + 13 ul 6x dye. Second line.
3. Cut out 7-10 kb gene.
4. Elute into Bio-rad - promega.

Fragment Isolation Protocol from Nu Sieve Gel.

1. Place the electrophoresed gel on the UV transilluminator and view under long wave light. Locate the fragment of 7-10 kb and cut out the band using a glass cover slip and place the gel fragment into a 1.5ml microcentrifuge tube.
2. Heat to 65°c until gel slice melts
3. Add 3 volumes (120 ul) of BioRad DNA isolation binding buffer and for every 2 ug of DNA add 5 ul (30 ul matrix) of matrix
4. Attach a clean 3cc syringe to a promega spin column and add the solution to it.
5. Push this slowly through the column (1 drop/3 sec)
6. Detach column from syringe and remove plunger.
7. Wash with 2 mls of 95% isopropanol.
8. Spin at 12,000 rpm for 20 sec.
9. Dry Column in Hybridization oven for a few minutes (65°c in 1) at 60°c)
10. Add 30-50 ul of water or TE. wait 1 min and spin again at 12,000 rpm for 20 sec.
11. Run 10 ul of the eluant on a gel to check recovery.

5 N°3

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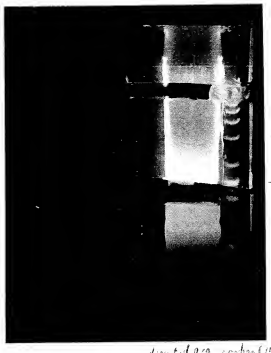
4 8

Notebook Number:

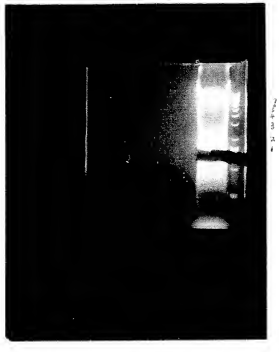
Date: 08/04/99



cut out 7-10 Kb

→ bright 1 Kb

digested with contain (1Kb)

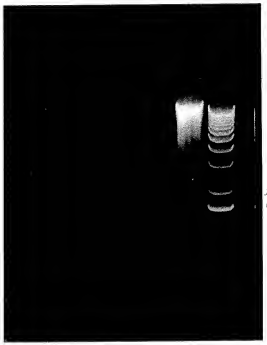


Do electrophorisis to check the result.

DNA 5ul + 1ul dye → 60v run

1.5ml Marker

Results:



Conjugation:

1. Donor Strain (E. Coli MGN617 PGP704 or MGN617 PLOF Km) are grown overnight with shaking at 37°C in 2ml LB broth containing 200 ug/ml ampicillin and 50 ug/ml kanamycin. (DAP)
2. Grown P. multocida 11039 overnight with shaking at 37°C.
3. 50 ul MGN617 ~~or 50 ul M~~ + 100 ul 11039 to 5ml of 10mM $MgSO_4$
4. Vortex for a few seconds.
5. Transfer to a 5ml disposable syringe and filter through a 25 mm Mitricel filter
6. Drain the filter and carefully remove it from the filter case with sterile forceps (curved tip work best)

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10 50 ul → [illegible]

100 ul IPTG
200 ul
+50 ul DAP

Notebook Number:
Date: 08/05/99

7. Place the filter on a LB plate cell side ~~up~~ up.
(avoid bubbles between the filter and the agar) PLOF
8 Incubate at 37 c for 8 - 18 hrs. 2.36V. Km

08/06/99

~~Results~~ 9. Resuspend the filter using 3 ml 10 m $MgSO_4$. Vortex.

PGP 704 BHI Ap 100 ul 10^0 } negative
BHI ApK 100 ul 10^0
BHI 100 ul 10^{-5}
LB ApDAP 100 ul 10^{-5}

PLOF Km BHI ApKm 100 ul 10^0 → negative
BHI Km 100 ul 10^0
BHI 100 ul 10^{-5}
LBApKm DAP 100 ul 10^{-5}
200 ul

Transformation by electroporation

PGP 704.

PLOF Km 10^0 10^{-1} 10^{-2} delution

Results ↓

08/09/99

Digest~~ion~~:

93-146 genomic DNA (cc pheol/ch— prep)

Leat incubate at 70°C 30min

Restriction Digest~~ion~~ EcoRI DNA 51 ul
10x buffer 6
EcoRI 3

37°C 4h. → put it in 65°C water bath

08/10/99

lb electrophoresis

903-146 genomic DNA (after digest) 60 ul

dye (6x) 10 ul

↓

run gel (marker 1kb 12 ul)

Fragment Isolation Protocol from Gel (See Page 7)

1. Cut out 7-10 kb and place the gel fragment into microcentrifuge tube
2. Heat to 65°C until gel slice melts
3. Add 3 volumes (630 ul) of BioRad DNA isolation binding buffer ~~resuspension~~ (1 ug ≈ 1 ul volume) and usually add 30 ul matrix.
4. Attach a clean 3 cc syringe to a promega spin column and add the solution to it.
5. Push this slowly through the column.
6. Detach column from syringe and remove plunger.
7. Wash with 2 ml's of 95% isopropanol
8. Spin at 12,000 rpm for 20 sec
9. Dry column in Hybridization oven for a few minutes.
10. Add 30 ul of TE wait 1 min and spin again at 12,000 rpm for 20 sec
11. Run Gel.

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9/~~29~~/99

Digest:

PID39	60 ul
Buffer	7 ul
EcoR I	3 ul

37°C 2h / 65°C 15 min.

9/30/99

1. Run a gel. 70 ul
2. Cut out 7-10kb.
3. Use another kit to extract DNA.

QIA quick Gel Extraction kit protocal (another kit)

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weight the gel slice in a tube. Add 3 volumes of Buffer QG to 1 volume of gel (100mg ~ 100ul)
3. Incubate at 50°C for 10min.
4. After the gel slice has dissolved completely, check that the color of mixture is yellow.
5. Add 1 gel volume of isopropanol to the sample and mix.
6. ~~To bind DNA, pipet the sample onto the QIA quick column~~ and apply vacuum. After the sample has passed through the column, ~~switch off~~ vacuum source.

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Run gel (Southern Blotting)

1. Digs EcoRI Sal I BamHI Xba I 1103E6k I ⇒ in back

2 ~~Rinse # Rinse~~

3. photograph with a ruler laid alongside the gel so that bound positions can later be identified on the membrane.

3 Rinse the gel in distilled water and place in a clean glass dish containing ~10 gel volumes of ~.25 M HCl. Shake slowly on a platform shaker for ~~3 min~~ 10 min at room temperature.

4. Pour off the HCl and rinse the gel with distilled water. Add ~10 vol denaturation solution and shake as before for 30 min to 1 hr.

5. Pour off the denaturation solution and rinse the gel with distilled water. Add ~10 vol neutralization solution. Shake as before for 30 min to 1 hr.

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11/19 20 199

Conjugation.

See P10 ?

put the conjugation in

① MGN 617 10^{-1} BHI Km

② 10^{-1} BHI Ap—

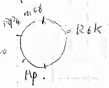
③ 10^{-1} BHI ApKm.

③ MGN 617 PGP 704

10^{-1} BHI Km

10^{-1} BHI Ap → ran grow

10^{-1} BHI ApKm



② MGN 617 PLOF Km

10^{-1} BHI Km X2 (100 ul. 20-30 colonies)

10^{-1} BHI Ap

10^{-1} BHI ApKm

10.0 ml 10^{-5} BHI 11039

10^{-5} LB DAp ApKm → MGN 617

↓

200 ml DAP

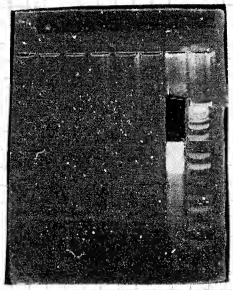
11/23/199

Cut out 2-6 Kb.

7-10 Kb. and more than 10 Kb

Use Qiaquick gel extraction kit to extract DNA.

result:



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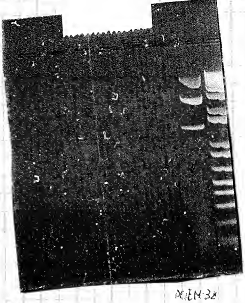

pGEM-32
plasmid DNA (1ul)

the digest ~~is not~~
is PGEM-32 has not been
digested completely.
So there are three bands.
(supercoiled DNA and one
strand DNA)
So repeat the digest

11/12/2000

11039 having 1 week long can not grow in BHI broth.
but can grow in BHI plate.
Inoculate 11039 and HGW 617. PGN 704. PLOFKm again
Do digest again 20 ul DNA (PGEM-32)
1ul EcoRI + 3ul buffer
over 2h. + 15' 65°C

11/13/2000

Sup 1ul + 4ul buffer → 37°C 2h. → 15' 65°C
+ ~~70%~~ 90ul 100% EtOH + 4ul 3M NaAc → frazor
+ ~~70% 100ul~~ 13500 rpm 15' → pour off supernatant →
100ul 70% EtOH 13500 rpm 10'. → pour off supernant
→ dry → run a gel (1ul)

Conjugation : LB plate + 200 ul DAP + 100 ul IPTG.
do conjugation

BHI → BHI + 100ul Ap → BHI Ap
BHI + 100ul Km → BHI Km
BHI + 100ul Ap + 100ul Km → BHI Ap Km

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2/4/00

Run a gel ~~Digest~~. 21.5 ml DNA
2.5 ul buffer overnight (3/07)
1 ul EcoRI

→ 65°C 15'

Run a gel. cut out 2–6 kb. 6–10 kb. morethe10
(because the extraction DNA always is bigger than
it is. so. cut out was smaller band)
Little

2/7/00

extraute DNA. (use Qiaquick Spin Handbook kit)

2/8/00

Run a gel. but no DNA

improve: Clean DNA befor

clean DNA

2/10/00

* Clean DNA
1/10 volume 3M Na acetate
2 vol 100 % etoh
→20°C ≥ 1h.
centrifuge ≥ 13000 rpm 4°C 15'
Remove supernatant
Add 100 ul 70% etoh
centrifuge ≥ 13,000 rpm 4°C
10 min
Remove supernatant. air dry ~ 5 min.
Resuspend 30 ul DNAse free H2O
Run a gel → cut out

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2/13/00

1. Run a gel (genomic DNA 11039)
2. Do digest

~~No~~ 1st has a little DNA may because the DNA doesn't ~~beer~~ dissolve well.

3/14/00

Set up PCR reaction

	①	②	③	④
primer SC 1011	0.8		0.8	0.8
primer SC 1012	0.8		0.8	0.8
dNTP	4	4	0	4
3.3x buffer	6	6	6	6
Mg	2.4	2.4	2.4	2.4
H_2O	6	7.6	10	6
	20ul	20ul	20ul	20ul
higher layer control DNA	0	0	0	0.5
3.3x buffer	9	9	9	9
enzyme	1	1	1	1
H_2O	20	20	20	19.5
	30 ul			

put 11039 in 56° for 2 hrs more than 3/14/00 because a lot of DNA do not dissolve in TE buffer.

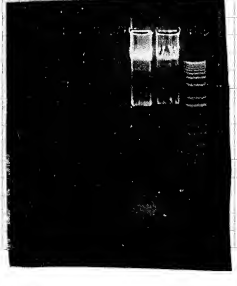
Run a gel (11039 genomic DNA.

① Line 1 ul DNA)
② Line 3 ul DNA.)

Do digest (11039) 75 ul total

62.5 ul DNA
7.5 ul buffer
5 ul Enzyme
} 37° overnight

65°C 15'



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in 3 sq rubber maid dishes put 2 pcs chromotograph filter paper (this is too soak up buffers)

1) Denaturation solution – takes very little just enough to soak up filter. 5 min.

2) Neutralization buffer 5 min.

3) 2x SSC 5 min. place it on the paper and dry for 5'
cross link on program c3 → put them in plastic.

4/13/00.

1. preheat the hybridization buffer to 42°c

2. In a suitable container prewet the blot in 5xSSC. Loosely roll the blot and place inside the tube. Add a small amount of 5xSSC to the tube and "unroll" the blot ensuring no air bubbles are trapped between the membrane and the tube. Don't allow the blot to overlap itself

3. Pour off the 5xSSC and add the appropriate volume of ~~the~~ hybridization (20ul). buffer.

4. prehybridize in oven for at least 30 min at 42°c.

5. prepare the labbled nucleic acid probe as instruction P13.

① Dilute the DNA to be labelled to a concentration of 10 ng/ul using the water supplied.

② Denature 100ng of the DNA sample (10ul) by heating for 5 min in a boiling water bath. (try 2ul + 8 ~~3 ul dam~~ gene + 7 ul H_2O) 10 – 2 – (damgene)

③ Immediately cool the DNA on ice for 5 min. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.

④ Add an equivalent volume of DNA labelling reagent (10ul) to the cooled DNA. mix gently but throughly

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5/4/06

prepare sequencing for genomic DNA.

Set up sequence program. (Liang sequence)

	3.2 dye	3.2 3mer primer	purified gen DNA	H2O
		(3 pmoles)	11839 bue	5ul
Roe-Lab 1x reaction	8ul	1ul (3 pmol/ul)		

total 20 ul

P.S. primer (add 3 ul primer to 97 ul H2O)

HAPDAM P2

HAPDAM M2

PCR

5/12/06 prepare sequencing

1. Column Hydration

① Remove the top of column, then add 0.8 ml of reagent grade water. invert for a few times, make sure no bubbles. leave the column for at least 2 hrs. at room temp.

② 2. Removal of Interstitial Fluid:

① remove the top cap first, then remove the end stopper from bottom

② allow excess column fluid to drain into a wash tube discard this fluid.

③ spin the column and wash tube in centrifuge at 750g for 2 mins discard it (collection tube)

3. Sample processing:

① Add 20 ul sequence(PCR) production into ~~tube~~ column (it's on another tube) make sure it's be into the column, but don't touch the column.

② spin the column at 750g for 2 min - the purified sample will collect in the collection tube

③ Dry the sample in a vacuum centrifuge

4. ① vortex resuspend sample in 25 ul of template reagent in

② vortex (30sec)) X2 2 min denature in 95°C

quick spin) Chill 5 min

put 10 ul in sequencing tube

freeze remaining (5 ul) at -20°C

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1180 colonies/ml => 1780000 colonies/ml

$\frac{1.78 \times 10^5}{0.005\ ug} = 3.56 \times 10^7$ / ug DNA

Result: the problem is xgal. the old xgal is not useful for producing blue colonies

Do electroporation again. and inoculate the restreaked bacterials to the LB Broth.

6/13/00

Extract the plasmid DNA (restreaked Bact.)

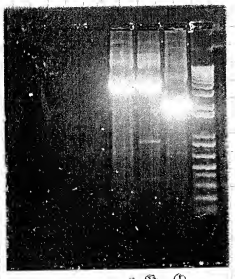
put the electroporation to refrigirator

Run a gel (plasmid DNA ~~EE~~ ① ② ③)

↓

2ul + 3ul + 1ul dye

DNA H$_2$O



③ ② ①

③ and ② is defenitely inserted. Do the digest.

20 ul. total

1 ul DNA

2 ul buffer

0.5 EcoRI

16.5 ul H$_2$O

overnight 37° load 3-4 ul on a gel.

6/14/00

Do ~~electroporation~~. southern hybridization:

Run a gel (plasmid DNA EcoRI digested. (20 ul)

load 3-4 ul per well.

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☆ 2 x SSC 5 min x 2 at room temp

5 x SSC 0.5% SDS 42°C 30' 轻 oven
伴以振荡 50 ml [illegible] 2.0 ml 10%

2x SSC 用软纸 (Kimwebs) 将膜的正面擦.

2 x SSC 洗干净 rinse and shake 5' x 2 min

7/9/00

Do Ligation
1 ul PGEM-3Z EcoRI + Sap trt.
8 ul insertor (11039 3-5kb extraction)
1 ul buffer
0.5 ul Ligase (New England Lab)

16°C overnight

7/10/00 65°C 15'

Do electroporation.

Note: cuvette should be chilled 5 min on ice
immediately add 1 ml LB medium and transfer
to a sterile culture tube.

12:10

Run a longer gel for 11038 EcoR I.
1 lane: ladder (2ul) 5:30 pm. 20V.
3 lanes: 11039 EcoRI.
5 " : ladder (4ul)
It goes very slowly. I should run this gel at 30V.
12:45 end.
1:10 continue
6:40 end

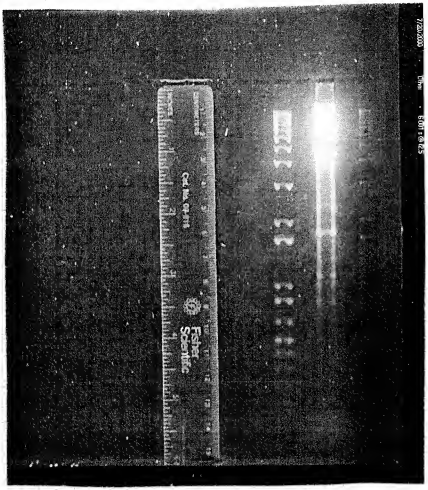
gel size 6 X 15

paper towel 8 x 15

filter paper 3 (5.5 x 14)

filter paper 5 (8x15)

membrane 7x14



the part from 6kb to 8kb has been separated. Do Southern hybridization.

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Make hybridization buffer.
100ml buffer
5 g blotting reagent
0.2 ml NaCl 2.925 g.

room temp. stir for 1 hr.
then 42°C at least 30'.

8/03/00
Continue Southern hybridization and Sequencing.

8/04/00
Sequencing didn't work again, but Southern hybridization did work.
set up the PCR reaction to make more 1-10-2 and check the plasmid DNA.

primer dilution
95 ul H_2O
2.5 ul 1Hpalam111 → stock for PCR reaction
2.5 ul ~ -- p1
100 ul
↘ 1ul.

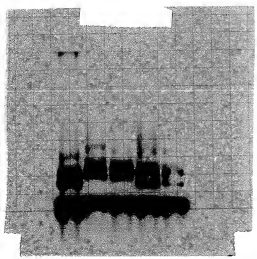
PCR reaction

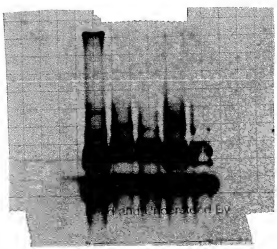
	1-10-2	0.5
	dNTP	5
	primers	1
	buffer	5
	taq	0.5
	H_2O	~~38.5~~ 37.5

	1-8-1	2
	dNTP	5
	primers	1
	buffer	5
	taq	0.5
	H_2O	36.5

	plasmid	1 ul
	dNTP	5
	primer	1
	buffer	5
	taq	0.5
		37.5

	Control	0
		5
		1
		5
		0.5
		38.5





8/25/00

Run a sequencing reaction

big dye	3 ul
plasmid	2 ul
primer	2 ul
H_2O	8 ul

Run a gel for genomic DNA (5% comp) in order to see if ladder

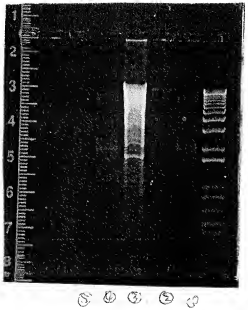
Should keep ~~some~~ a little 11039 Hind III cut with left for Southern in vivo region

8/28/00

Run a gel. 3 ul for extracted DNA
3 ul for EcoR I 11039 digest (positive control)

① extracted DNA 4-6 kb
② " " 6-9 kb
③ 11039 EcoRI
④ repeat ② → another tube
⑤ repeat ③ → another tube

8/29/00



⑤ ④ ③ ② ①

the size of both tubes is bigger than needed. So extract DNA from 3-4 gel slice.

8/30/00

Run a gel

10/9/00

Digest : Clone -a- 1- a plasmid DNA to get probe

3 ul 3 ul DNA
Digest 3 ul buffer
1 ul ~~EcoRI~~ ~~HindIII~~ EcoP I
~~23 ul~~ H2O

30 ul --> 2h 37°C
15' 65°C

cut out from 1.4 kb.

PGEM-3Z hindIII.

10/10/00

1. Continue plasmid DNA +sap -treatment
2. do gel extraction.

10/11/00

Run a gel for gel extraction (for probe) and PGEM-3Z hindIII + sap trt. (both are seed)

gel extraction (probe) just need to use 3ul for detect.

I use PBS 1.5 ul
Insert 7.5 ul
buffer 1 ul
Lysse 0.5 ul

10/12/00

the electroporation didn't work. try electroporation. 1 ul PGEM3Z + [illegible]

↓

LBAp 10^{-2}. 10^{-4}.

to

10/13/00 it did work.

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digest

	①	②	③
DNA	1	1	1
buffer	5	5	5
EcoRI	0.5	0.5	0.5
H_2O	43	43	43

50 ul

2/31/01

Do pcr again

	①	②	③
P multocida A:3	0	1	1
dNTP	5	5	5
primer	1	1	0.5
buffer	5	5	5
New tag	0.5	0.5	0.5
H_2O	38.5	37.5	38.0

50 ul

95°C 2min

75°C 30sec. 64°C 1min 72°C 2min 1 cycle

72°C 10min 35 cycles

4°C. Hold

8 ul

there're a little DNA on the gel. It's not clear. So Add more genomic DNA to do PCR again

2/01/01

Do PCR again. Add more genomic DNA and increase ~~decrease~~ annealing temperature to 66°C.

	①	②	③	④
P mul (A:3)	0	3	6	10
dNTP	5	5	5	5
primer	1	1	1	1
buffer	5	5	5	5
Tag	0.5	0.5	0.5	0.5
H_2O	38.5	35.5	32.5	28.5

50 ul

annealing temperature 66°C 1'

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3/5/01

1. Run a gel for H digest.

2. Do a Klenow for clone A ~~EE~~ cla I and T-hIII I digest

Klenow: 20 ul digest (the concentrate of my DNA is high enough)

0.8 ul dNTPs (200 ul of each in working solution)

1 ul 0.1 mg/ml BSA (I skip this step)

0.5 ul klenow

I didn't add buffer because the Multi Core (buffer) is compatible for klenow

incubate @ 37°C for 30 min. HI @ 75°C for 10 min.

(I lost this Klenow product)

3/6/01

Do digest for B. C. E. F. G. H. and Rnd a gel for it.

I select B. C. G to run a seq. reaction again. And to make sure which one is right.

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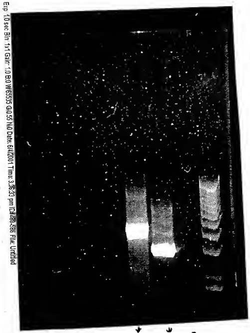


PPS88 ↔ MGN617

6/5/01.
Do the digest for PPS88 (Xba I + Kpn I)

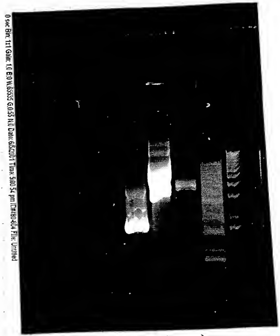
5 ul DNA
12 ul H_2O
(multi core) buffer 2 ul
0.5 ul XbaI
0.5 ul kpnI

2h 37°C

inactivate 65°C 15'

Run a gel for digestion, genomic DNA → PCLPm2. (from clone A) and pbluescript plasmid

all the samples → 1 ul



PPS 88 clone A plasmid MGN.

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do sequencing react: for number 5

Run a gel for ① ② ③

big aye 8 ml
plasmid 2 ml
primer T3 2 ml
water 8 ml

Sample	Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
bluescript and dam①	dsDNA	3.631	5.554	3.175	0	1.74929	0.2777 ug/uL	1	50	174.93

↓ number 5.

6/29/01

Sample	Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
pLS88	dsDNA	0.577	1.176	0.606	0	1.94059	0.0588 ug/uL	1	50	194.06

8 ml PLS 88
XbaI / BamHI 0.5 ml
buffer (multicore) 1 ml
water 0.5 ml

8 ml PLS 88
Xba I 0.5 ml
BamH I 0.5 ml
multicore 1 ml

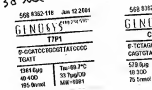
7/3/01

PCR:

				(control)
Template	pcLpm3	0.5 ml	1 ml	0
dNTP		5 ml	5 ml	5 ml
primer		1 ml	1 ml	1 ml
buffer		5 ml	5 ml	5 ml
Taq		0.5 ml	0.5 ml	0.5 ml
H2O		38 ml	37.5 ml	38.5 ml

primer dilution

95 ul	H2O
25 ul	T7P1
25 ul	CLdam M20



GENOSYS
568

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8/15/01 Cont.

PCR4 XL1B electroporation . . .

Blotted plates with Hybond membrane

followed protocol for colony Hybridization

Cross linked Membrane -- stored at -20C ON

3/16/01

Colony Hybridization PCR4 XL1B

made probe using 3ml PCR-4 + 7ul 1620 in ECL Kit

preheat Hybridization buffer to 42°C

wet Screen mesh + Membranes
Roll + put in hybridization tube
pre hybridize with SSC for 1 Hour
5X
use 30mls hybridization Buffer

Probe Boil 5"
Cool on ice 5"
10 ul labelling reagent
10 ul glutaraldehyde
10' 37°C
add 1ml hybridization buffer from tube - add buffer + probe to tube 42°C ON

Friday
8/17/01

Colony Hybridization Cont.

preheat 1° wash buffer to 42°C
discard Hybridization buffer. ~~Add Scrub~~
Add 50ml 5X SSC return to Oven 5min
discard 5X SSC add 1/3 vol of tube w/1° Wash Buf
Return to Oven for 20min
Wash again in 1° wash buff for 10 min 2X
remove blots from tube place in glass container
Cover w/2X SSC
Shake @ RT for 5min X2

Detection
8ml/membrane 13mls #1 13mls #2 3membranes
incubate 1min
place on Saran

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Notebook Number: _____

Date: _____

9/19/01 Cont.

Culture reached 260 KU after 4 hrs incubation at 37°C

(2) Put cultures into 8534 tube

(3) Incubate on ice 15"

(4) Cf 4°C 5000 x g (6500 rpm) 15"

(5) Wash 2X w/ 1mM Hepes buffer (pH 7.0)

(6) resuspend pellet in 10% glycerol to 1/12 their Original Vol = 2 mls

(7) Cf 5000 x g 10"

(8) resuspend in remaining glycerol solution after supernatant is decanted

(9) Flash freeze in EtOH + dry ice bath

(10) Freeze at -80°C

P. mult 11039 is highly encapsulated + forms a very soft pellet I lost the pellet in the 1st wash step, so more cells will have to be prepared

Electroporate pLS88 into P. mult 1069

1 ul pLS88 plasmid prep (in plasmid box spot G9)

40 ul 1069 comp cells

2.5 KV
200 Ω
25 uF
Tc = 5.12 msec

RESULTS

10^{+1}	134 colonies
10^{0}	2
10^{-1}	0

Incubate 37°C 1 hr in LB

plate 10^{0}, 10^{-1}, 10^{+1} 100 ul on LB strep 37°C

10^{+1} = after plating 10^{0} + diluting 10^{-1}, Cf culture + resuspend pellet in 100 ul Media - plate 100 ul

9/25/01 Tuesday

Pass colony from R6E12 #4 blot - do a plasmid prep

Electroporate pLS88 into P.mult 11039

1 ul pLS88 plasmid prep (in plasmid box spot G9)

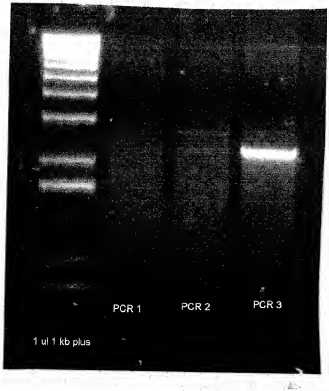
40 ul 11039 comp cells (flash-frozen in liq N2)

2.5KV
200 Ω
25 uF
Tc = 5.1 to 5.2 msec

RESULTS
10^{+1} 80
10^{0} 13
10^{-1} 1

Incubate 37C 1 hr LB

Plate 10^{0}, 10^{-1}, 10^{+1} 100ul on LB strep 37°C



See pg. 35 for PCR rx setup

Annealing temp = 48°C

Extension time = 30 sec

pcr rx did not work again

0.7% agarose 3.5ul gel star
5ul of PCR Rx loaded

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Notebook Number: _____

Date: _____

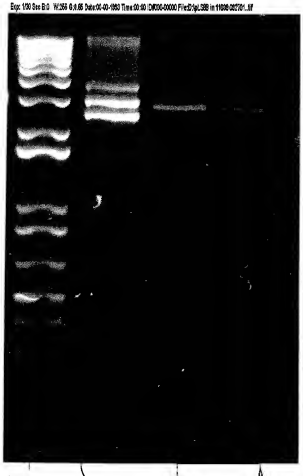
9/26/01 Wednesday - Gone to Memphis

ML started 5ml culture of ~~pMBP1 E.i.2~~ R6Ei2 Ed 9+

9/27/01 Thursday

Did a Qia Spin mini prep of R6Ei2

Eluted in 30ul EB



1ul 1kb Plus | R6Ei2 1ul | PLS88 in P.mult 11039

R6Ei2 looks good

Pass to a new plate

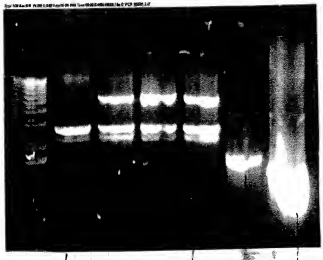
freeze next week

PLS88 in 11039 looks good -

it work - we were abbles to electroporat into P.multocida Comp cells

PCR pCLpm3 T7P1 + CLdamM20 092801			
Sample #	1	2	3
pCLpm3 8/29/01 BYR	0.5	1	0
dNTPs	5	5	5
T7P1 + CLdamM20	1	1	1
Taq	0.5	0.5	0.5
Taq Buffer	5	5	5
Water	38	37.5	38
TOTAL Rx amt	50	50	
Annealing temp	67.5 C		

10/2/01



PCLpm3 1ul

See pg 41 pcr Set up

10ul cut out of gel = 0.4177 gm put in Ref over night

10/3/01

Elute DNA from gel with Qiagen gel extraction Kit

cut gel fragment in ½ and put in 2 tubes
Added 600ul / tube of QG
Added 200ul isopropanol

eluted each column w/ 30ul EB into same tube = 60ul total

Called pCLpm3 PCR

Loaded 5ul of pclpm3 PCR on 0.7% agarose gel 3.5ul gelstar
100 Volts

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Notebook Number: _____ Exhibit A Page 36

Date: _____

10/3/01

Electroporated 40 ul P. multocida 11039 Comp Cells w/no DNA

Recover 1 hr in LB broth 37 C

2.5 KV
25 uF
200 Ω
Tm = 5.16 msec

plate 100 ul 10^{0}, 10^{-1}, 10^{-1} on LB grow at 37 C ON

Should have plated on LB strep — threw plates away

Before Digesting pCLpm 3 PCR 1.7 Kb frag we need more PCR product b/c XbaI has to be deactivated w/ EDTA (heat does not work) + then the DNA is ppct out. DNA is lost in ppct so I need to amplify my PCR product

PCR pCLpm3 1.7 kb	T7P1 + CLdamM20		10/3/01
Sample #	1	2	3
pCLpm3 PCR 1.7 kb	0.5	1	0
dNTPs 1mM each	5	5	5
T7P1 + CLdamM20	1	1	1
Taq (7/12/01)	0.5	0.5	0.5
Taq Buffer	5	5	5
Water	38	37.5	38.5
TOTAL Rx amt	50	50	50
Annealing temp	67.5 C		
extension time	30 sec		

run 0.5 4ul per rxs on gel

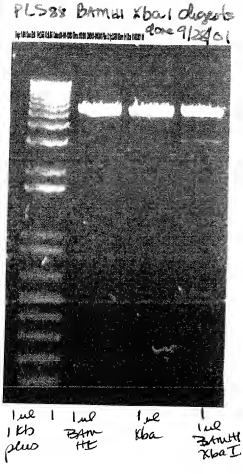
Read and Understood By

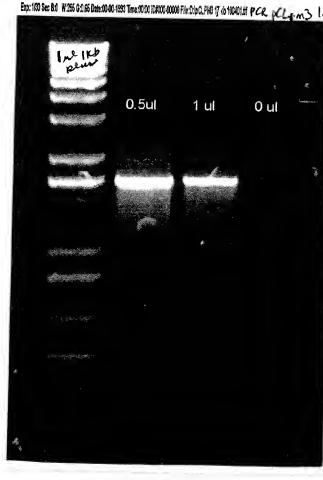
pLS88 digest BamHI/Xba I

10ul pLS88 plasmid prep 9/19/01
1ul Xba I
1ul BamHI
2ul Multicore Buffer 10X
6ul H2O
20ul

Digest 37C 4 hrs start 10:00am

Run 0.5ul of pCLpm3 1.7Kb pcr Rx run 10/3/01 on 0.7% agarose gel 3.5ul gel star





Read and Understood By

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Signed

10/4/01

Digest pCLpm3 1.7Kb frag pCR Rx 1 (from 10/3/01)

10 ul	DNA	
1 ul	Xba I	37 C
1 ul	BamHI	
2 ul	MultiCore 10X Buffer	4 hrs
6 ul	H2O	
20 ul		

To pUC88 + pCLpm3 1.7Kb BamHI Xba I digests

Add 0.5ul 0.5M EDTA to deactivate Xba I
Add 2 ul 3M Na Acetate (1/10 vol)
44 ul ETOH (2 vols)

freeze -20 overnight to ppct DNA.

Start 2 1L BHIAp cultures of R6Ei2 for LRS prep
Add 2.5mls of ON culture per Liter
Add 500ul 200mg/ml Ap = 100ug/ml final vol
1/2 normal conc. used
grow ON 36°C Shaking

10/5/01

Cf. R6Ei2 cultures in 250ml bottles in GSA rotor
6000 rpm for 15min at 4°C
freeze pellets at -20 C.
Do LRS prep next wk

10/5/01

Cf. ppt DNA pLS88 + pCL PM3 1.7Kb resuspend in 10 uL TE. put at 4C to go into suspension

10/8/01

Run 1ul pLS88 + pCLpm3 1.7Kb on gel
✓ on gene spec first

Sample	Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
pCL PM 3 1.7 kb	dsDNA	0.655	1.536	0.818	0	1.87775	0.0768 ug/uL	1	50	104.32
	dsDNA	0.707	1.592	0.85	0	1.87294	0.0796 ug/uL	1	50	104.05
pLS88 Bam HI Xba I	dsDNA	1.565	3.982	2.369	0	1.68088	0.1991 ug/uL	1	50	93.38
	dsDNA	1.987	4.493	2.85	0	1.57649	0.22465 ug/uL	1	50	87.56

pCl pm 3 76.8 ng/ul pLS88 211.9 ng/ul

Ligation

pLS88 4.5 Kb
pCL PM 3 1.5 Kb

pLS88	1ul	200ng
pCL Pm3	7.5ul	570ng
Ligase Buff	1ul	
Ligase	0.5ul	
	10ul	

14°C overnight

10/9/01 Tuesday

Heat inactivate ligation 65°C 15'

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PCR 93146 Ei OPS primers 10/17/01

Sample #	1	2	3	4
93146 genomic prep 7/28/99	2	2	2	2
dNTPs 2.5mM	10	10	10	10
Ei OPS PCR U 2 + Ei OPS PCR R 2	1.2	1.2	1.2	1.2
EXL polymerase	1	1	1	1
DMSO	0	0.5	1	1.5
Stabilizing Soln	1	1	1	1
10 X Buffer	5	5	5	5
Water	29.8	29.3	28.8	28.3
TOTAL Rx amt.	50	50	50	50
Use PCR EXL Pcr Program				
Annealing Temp	63			
PCR EXL primer				

Ran out of EXL polymerase after 1st Rx.
Ran 1st Rx ordered more EXL poly
Rxs 2-4 are in green ice box -20 chest freezer

gel picture pg 54

Make 2 5ml LBAp cultures of pFPV25 in DH10Bs
1 - plasmid prep
1 - freeze

Make 5ml culture of pLS88 isolates pos + Adj in LBstrep
positive colony from ~~blot~~ hybridization ✓ adjacent to positive colony

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Signed

10/18/01

Started cultures in wrong Antibiotics
started pFPV25 in LB strep · pLS88 ~ LBAp Opps -

Start new 5 ml cultures
pFPV25 in LB Ap
pLS88 in LB Strep

10/19/01

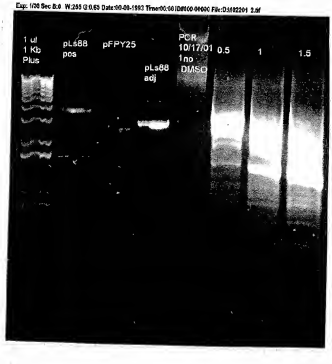
Froze 1 pFPV25 culture (Ask B/L where it is)

Spin down other pFPV25 culture + both pLS88 cultures (pos + adj) - freeze pellets for plasmid preps

10/22/01

made Qiaspin plasmid preps of

PFPV25, pLS88 positive · pLS88 adjacent



Loaded 1 ul of
pLS88 pos
pLS88 adj
pFPV25

Loaded 5 ul of
pcr products

pass both pLS88 colonies
to A new LB strep plate.

Read and Understood By

10/23/01

Digest pLS88 pos + Adj plasmidpreps (10/22/01)

Confirm insert

pLS88 pos	4 µl	pLS88 Adj 4 µl
BamHI	1 µl	
Xba I	1 µl	
multicore	2 µl	
H2O	12 µl	
	20 µl	

BamHI/Xba I cuts the insert out

load 10 µl on gel

Linearize

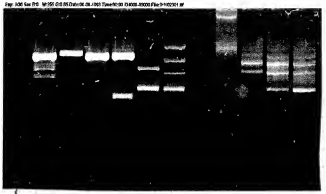
pLS88 pos	2 µl	pLS88 Adj	1 µl
EcoRV	1 µl	EcoRV	1 µl
Buff D	1 µl	Buff D	1 µl
H2O	6 µl	H2O	7 µl
	10 µl		10 µl

load 10 µl on gel

Linearize DNA

pFPV25-	5 µl	pFPV25-	5 µl
EcoRV	1 µl	pst	1 µl
Buff D	1 µl	Buff H	1 µl
H2O	3 µl	H2O	3 µl
	10 µl		10 µl

load 10 µl on gel



1 KB plus did not show up

1) pLS88 adj BamHI/XbaI 10 µl + 2 µl 6X Dye
2) pLS88 pos EcoRV
3) pLS88 Adj EcoRV
4) pLS88 pos BamHI/Xba I
5) pFPV25- pst I
6) pFPV25- EcoRV
7) PCR 10/17/01 No DMSO 2 µl DNA + 1 µl 6X dye + 3 µl H2O
8) 0.5 µl
9) 1 µl
10) 1.5 µl

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Signed

Notebook Number: _____ Exhibit A Page 43

Date: _____

10/24/01

PCR pMBEi1 Ei OPS primers 10/24/01				
Sample #	1	2		
From 9/7/00 in MBEi1 DNA Box pMBEi1	0.5	0.5		
dNTPs 2.5mM	10	10		
Ei OPS PCR U 2 + Ei OPS PCR R 2	1.2	1.2		
EXL polymerase	1	1		
DMSO	0	0.5		
Stabilizing Soln	1	1		
10 X Buffer	5	5		
Water	31.3	30.8		
TOTAL Rx amt.	50	50		
Use PCR EXL Pcr Program				
Annealing Temp	63			
PCR EXL primer				

pLS88 positive gene size

Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
dsDNA	3.461	5.406	4.733	0	1.14219	0.2703 ug/uL	1	50	83.46
dsDNA	0.046	1.798	1.112	0	1.61691	0.0899 ug/uL	1	50	89.83
dsDNA	0.018	1.781	1.091	0	1.63245	0.08905 ug/uL	1	50	90.69
dsDNA	0.031	1.779	1.083	0	1.64266	0.08895 ug/uL	1	50	91.26
dsDNA	0.3	2.076	1.421	0	1.46094	0.1038 ug/uL	1	50	81.16
dsDNA	0.732	2.531	1.855	0	1.36442	0.12655 ug/uL	1	50	75.80
dsDNA	0.538	2.383	1.707	0	1.40187	0.11965 ug/uL	1	50	77.88
dsDNA	0.37	2.2	1.522	0	1.44547	0.11 ug/uL	1	50	80.30

pLS88 plasmid prep made 10/24/01 Conc = 134.8 ng/ul

Seq pLS88 w/ CLPM1-PL 3.2pmol → p.29 blr + w/ T3 3.2pmol → pg 27 bkV

pLS88 4ul
TRR 8ul
primer 2ul
H2O 6ul
20ul

#1 CLPM1-PL
#2 T3

Sequence should contain DAM gene sequence
CLPM1-PL primer sequence data is trashy - but
I blasted a segment of it and it is the DAM gene
sequence

Redigest pFPV25 5ul
Buff H 1ul
H2O 3ul
ECORI 1ul
~~Water~~ ~~Bulk~~
10ul digest

Pst I 1ul

pFPlas 5ul
ECORI 1ul
pst I 1ul
Buff H 2ul
H2O 10ul
20ul

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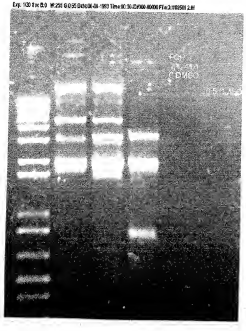
Signed

Notebook Number: _____

Date: _____

10/25/01



0.7% agarose 3.5 ml gel stain

1) 1 µl 1 Kb plus + 4 µl H_2O + 1 µl lox dye
2) pFPV25 EcoRI 10 µl + 2 µl
3) pFPV25 Pst I 10 µl
4) pFPV25 EcoRI/Pst I 10 µl
5) PCR pMBEI 0.5 µl No DMSO
6) " " 0.5 DMSO

+ 1 µl 6X dye
3.5 µl H_2O

Freeze pLS88 pos ~~xxxxxxxx~~
Start 5 ml LB strep

Electroporate pLS88 pos into P. mult 11039 comp cells

2.5 KV
200 Ω
2.5 µF
Tc = 5.08 ms

40 µl P. mult (All D4 9/21/01)
1 µl pLS88
Recover 1 hr LB strep

plate 10^{+1} on LB strep
Incubate 37 °C ON

10/26/01

Make Qiaspin plasmid prep of pFPV25 in DH10βs
Elute 30ul EB

Digest w/ EcoR1 / Pst 1 (same as yesterday)
digest 5 hrs

run on gel w/ undigested plasmid

Freeze Pls 88 pos now called pls 88 DAM
it is in XL1B MRF'
All D5-9 LBstrep + 20% glycerol

Put plate from Electroporation in Ref over wkend - Monday pick 4 big colonies + plate on LB strep

Started BH1 Ap plate cultures of 93146 W7 Lux + 93146 Rw Lux

Imaged fish immersion + ip injected w/ 93146 W7 Lux on Night Owl.

Luminescent area

Read and Understood By

Signed

Signed

pLS88 positive gene size

Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
dsDNA	3.461	5.406	4.733	0	1.14219	0.2703 ug/uL	1	50	83.46
dsDNA	0.046	1.798	1.112	0	1.61691	0.0899 ug/uL	1	50	89.83
dsDNA	0.018	1.781	1.091	0	1.63245	0.08905 ug/uL	1	50	90.69
dsDNA	0.031	1.779	1.083	0	1.64266	0.08895 ug/uL	1	50	91.26
dsDNA	0.3	2.076	1.421	0	1.46094	0.1038 ug/uL	1	50	81.16
dsDNA	0.732	2.531	1.855	0	1.36442	0.12655 ug/uL	1	50	75.80
dsDNA	0.538	2.383	1.707	0	1.40187	0.11965 ug/uL	1	50	77.88
dsDNA	0.37	2.2	1.522	0	1.44547	0.11 ug/uL	1	50	80.30

pLS88 plasmid prep made 10/24/01 Conc = 134.8 ng/ul

Sq pLS88 w/ CLPM1-PL 3.2pmol → p.29 blr + w/ T3 3.2pmol → pg 27 bkV

pLS88 4ul
TRR 8ul
primer 2ul
H2O 6ul
20ul

#1 CLPM1-PL
#2 T3

Sequence should contain DAM gene sequence
CLPM1-PL primer sequence data is trashy - but
I blasted a segment of it and it is the DAM gene
sequence

Redigest pFPV25 5ul
Buff H 1ul
H2O 3ul
EcoRI 1ul
~~Blank~~
10ul digest

Pst I 1ul

pFPlas 5ul
EcoRI 1ul
pst I 1ul
Buff H 2ul
H2O 10ul
20ul

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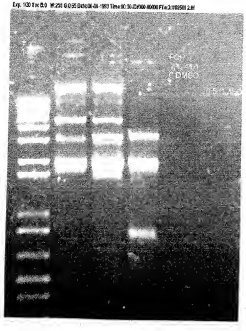
Signed

Signed

Notebook Number: _____

Date: _____

10/25/01



0.7% agarose 3.5ml gel stain

1) 1ul 1 Kb plus
+4ul H_2O
+1ul lox dye
2) pFPV25 EcoRI 10ul + 2ul
3) pFPV25 pst1 10ul
4) pFPV25 EcoRI/pst1 10ul
5) PCR pMBE1 0.5ul No DMSO
6) " " 0.5DMSO

+1ul 6X dye
3.5ul H_2O

Freeze pLS88 pos [redacted]
Start 5ml LB strep

Electroporate pLS88 pos into P.mult 11039 comp cells

2.5 KV
200 Ω
25 uF
Tc = 5.08ms

40ul P.mult (All D4 9/21/01)
1 ul pLS88
Recover 1hr LB strep

plate 10^{+1} on LB strep
Incubate 37°C ON

10/26/01

Make Qiaspin plasmid prep of pFPV25 in DH10βs
Elute 30ul EB

Digest w/ EcoR1 / Pst 1 (same as yesterday)
digest 5 hrs

run on gel w/ undigested plasmid

Freeze Pls 88 pos now called pls 88 DAM
it is in XL1B MRF'
All D5-9 LBstrep + 20% glycerol

Put plate from Electroporation in Ref over wkend - Monday pick 4 big colonies + plate on LB strep

Started BH1 Ap plate cultures of 93146 W7 Lux + 93146 Rw Lux

Imaged fish immersion + ip injected w/ 93146 W7 Lux on Night Owl.

Luminescent area

Read and Understood By

Signed

Signed

Notebook Number: _____

Date: _____

60 10/31/01 Pick 4 colonies from P.mult 11039 pLS88 electroporation. grow on LB strep

11/5/01

pour 12%/4% SDS-PAGE gel
gel stayed in buffer in 4°C ON

Start 5ml LB strep cultures of P.mult 11039 pLS 88 From 10/31

11/6/01

Samples to run on gel

LPS from R6Ei2 dilute - 1/1 in sample Buffer

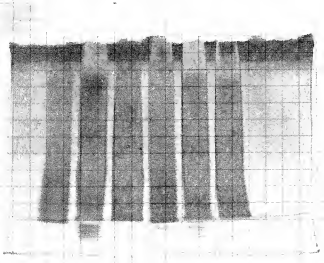
and take 10ul (1mg/ml) + 15ul H2O + 25ul Buffer = 10ug

LPS Salmonella typh 1/1 of 2mg/ml in H2O 1/1 in Buffer

Lane 1 Sal typ
2 R6Ei2 1/1 } Not Denatured
3 R6Ei2 10ug
4 Sal typ
5 R6Ei2 1/1 } Denatured
6 R6Ei2 10ug

loaded 10ul / well Ran at 100volts

Silver stain for LPS



Read and Understood By

Made Qiaspin plasmid preps of P.mult 11039 pLSS8 4 colonies

11/7/01 Run 12%/4% SDS PAGE gel
Same as 11/6/01
1) Sal. type 1/1 of 2mg/ml in H2O 1/1 in Buffer - Load 10 ul
2) R6E12 1/1 in buffer
3) R6E12 10ug
4) WT 93146 LPS 10ug
Electrophoresed at 100Volts
transfered to Nitrocellulose 100 volts 1 hr
Blocked Membrane overnight 5% NFM in PBS
Our Ed 9 was contaminated - so was Bobbie's

DH10Bs pFPV25 frozen -80C All F1-5
tube #1 used to start a 5ml LBap culture
& put on LBap plate

P.mult 11039 pLSS8 #1 Start 5ml LB strep culture to freeze → see gel pic Next page

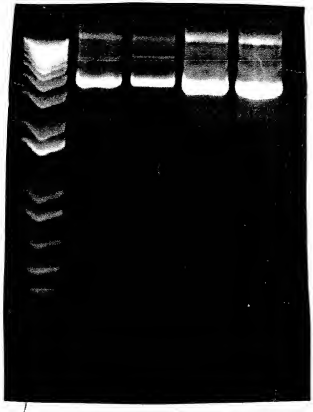
Read and Understood By

Signed

Signed

Notebook Number: _____

Date: _____



p. mut 11039
Dan gene pLs 88
plasmid preps
from 11/6/01

1 ml
1Kb plus

11/8/01

Wester Blot R6E12

There is No E8 9 to use as 1°Ab so

1° used α Ed ict 4383 polyclonal CF serum 1/1600
+ α Ed ict 9346 wt polyclonal CF serum 100ml added
to 1/1600 dilution

Incubate RT 1 hr shaking
Wash 3X PBS TW20

2° 9E1 1/4 1 hr RT shaking
wash 3X PBS TW

3° goat α mouse Ig AP 1/1500 1 hr RT Shaking
Develope BCIP/NBT 20 mins?

It did not work
will have to repeat
when Bobbie has more
Ed 9

1 2 3 4

Frozy pasturella multocida 11039 pls88 (DAM gene)
LB strep + 20% glycerol
-80 C Box A11 G1-5

Digest pls88 p.mult 11039 #1

BamHI/XbaI
DNA 4ul
BamHI 1ul
XbaI 1ul
multicore 2ul
H2O 12ul
20ul

BAM HI
DNA 4ul
BamHI 1ul
multicore 1ul
H2O 4ul

Digest 37°C
for 2 hrs approx
run on gel tomorrow

Plasmid prep pFPV25 in DH10Ba
Eluted in 30 µl set next pcy for gel pic

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Signed

64

Notebook Number: _____

Date: _____

11/8/01

pFPV25

do not know what this band is

next Run once and extract top band & electroporate into XL1B

93146 Lux recovered from fish immersion infected

Bacteria was Cultured 4 days post immersion.

Lux plasmid is Stable in vivo

Neg Controls 14 & 15

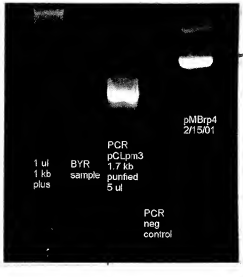
22 = 93146 Lux

Read and Understood By

PCR pCLpm3 1.7 kb T7P1 + CLdamM20 purified 01/16/02

Sample #	1	2	
pCLpm3 PCR 1.7 kb purified	0.5	0	
dNTPs 1mM each	5	5	
T7P1 + CLdamM20	1	1	
Taq (7/16/01)	0.5	0.5	
Taq Buffer	5	5	
Water	38	38.5	
TOTAL Rx amt.	50	50	
Annealing temp	67.5 C	program m11 under cheerk	
extension time	30 sec		

1/18/02
run 5ul on gel of PCR Rx 5ul DNA 1ul 6x Dye



measured pMBrp4 on nano spec - got a low reading of 11.8 ng/ul
So ran 1ul on gel -
Conc on gel looked like = 100 ng/ul

digest w/ BamHI HindIII

		double digs
pMBrp4	4 ul	4
enzyme	0.5 ul	0.5 each
10x Buff E	1 ul	1
H2O	4.5 ul	4

digest from 2:00 - 5:00 37°C

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Signed

2/13/02.

Ligation: To End Conversion Rx mix add:

(1) 1ul pts 88 Vector (pts 88 & EcoRV digest)
1ul ligase.

(2) + Control
1ul pT7 Blue Vector
1ul ligase

(3) - Control
1ul pT7 Blue Vector
1ul ligase

22°C. 15"

Transformation:

Add 1ul ligation Rx to 1 tube of Nova Blue Comp cells
Incubate on ice 5min
heat Shock 30 sec @ 42°C water bath
Incubate on ice 2 min
Add 250ul RT SOC media
plate

(+) + (-) Controls 50 ul @ 1/10 (5ul in 45ul H_2O)
on LB S-gal

exp 50ul @ 1/10 on LB strep

2/14/02

Colonies from ligation were very small first thing this AM.

Read and Understood By

Signed

Signed

Notebook Number: ______

Date: ______

3/14/02 Thursday

PCR pCLpm3 1.7 kb T7Sal I P2 + CLdamECoRV 3/14/02			
Sample #	1		
pCLpm3 PCR 1.7 kb purified (1/23/02)	0.5		
dNTPs 1mM each	5		
T7Sal IP2 +CLDamECoRV	1		
Taq (7/16/01)	0.5		
Taq Buffer	5		
Water	38		
TOTAL Rx amt.	50		
Annealing temp	55		
extension time	30 sec		

used wrong template
repeat using
pCLpm3 plasmid prep
8/29/01 red box G8

15 7369-006 Mar 7 2002
GENOSYS
CLDamEcORV
5' CTCTTTTGATATCGTCTCT TTTCA
341.6µg | Tm=60.3°C
10.0OD | 34.2µg/OD
47.3nmol | MW=7225

15 7369-005 Mar 7 2002
GENOSYS
T7SalIP2
5'-CCCCTGATTCTGTCGACA
514.3µg | Tm=62.4°C
15.4OD | 33.4µg/OD
95.1nmol | MW=5410

reconstituted primers
2.5 µl each + 95 µl H_2O

Made new 1mM dNTPs
40 µl 10mM mix to 60 µl H_2O

plated pLS58 from frozen stock A3 E4 put on LB strep at 37C. MLL will take out of incubator + put at 4 C tomorrow

Notebook Number: ______

Date: ______

4/16/Tue

Hybridize colonies from Nls5x Pcrp-n3 ligation

follow protocol pg 50

probe

pcR prod green pcR box A9 - pcR rx 3/21/02
pg 94 bk 1 = 535 ng/ul

Cleanup pcR prod using microcon columns - follow directs in kit

removes primers + dNTPs

Re read on Gene Spec -

Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor
dsDNA	0.709	0.366	0.224	0	1.63393	0.0183 ug/uL	1	50
dsDNA	0.736	0.655	0.497	0	1.31791	0.03275 ug/uL	1	50
dsDNA	0.492	0.945	0.804	0	1.17537	0.04725 ug/uL	1	50
dsDNA	0.551	0.997	0.861	0	1.15796	0.04985 ug/uL	1	50

there are crazy readings - gene spec is not good for measuring DNA of pcR prod.

MLL said use 3ul in probe

Remainder of cleaned-up pcR prod is in Pink Box 118

Hybridize ON 42°C

4/17/02 Developed Hybridization

1" film almost had no image
10" very light image
1 hr OK

Plate 1

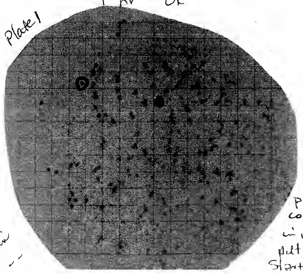
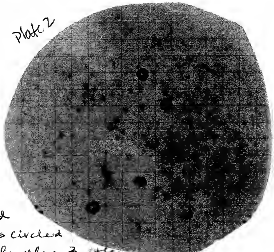


Plate 2



passed colonies circled in purple plus 3 other

put on LB strep

Start 5ul LB ...

plates in ...

5/13/02

cycle seq PGEM 32 test plasmid w/ M13 primer from ABI
Run on seq. to create Matrix file

5/14/02 Tues

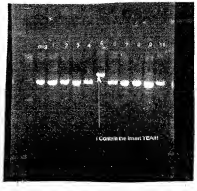
Clones from Electroporation grew well

grow 2ml LB strep cultures
pick colonies w/ sterile toothpick
use 1ml tomorrow to do plasmid preps to V for insert

grow 1 culture from Neg Control

pls88 pc/pm3	100ml	≈150	53
	50ml	26	5
Neg Con (pls88 No insert)	100ml	21	~~35~~
	50ml	14	11

pick 10 ligation colonies
1 neg con colony



5/15/02 Wed

All 2ml cultures grew

used 1ml to do Qiaprep spin plasmid preps

eluted in 30ml EB

Run 3ml DNA on gel
1ml 6X Dye
2ml H20
6ml

It Finally Worked!!

#5 & Neg plasmid preps in yellow box A5 + A6

For Vaccine trial:
plated 93146 R6 from 6/19/01 A4G5 -80C on Blood
grow at 36°C 48hrs

Reconstituted Intervet Edict vaccine - filled bottle 2/3 full w/ sterile H20

plate 1 loop full on Blood grow at 26 C 48hr
Put 50ml into 5ml BHI to freeze back grow at 26C shaking over night

1 750ml flask BHI for Intervet Vaccine
4 750ml flask BHI for R6

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8

Notebook Number: _____

Date: _____

For Vaccine trial cont:

Took 5mls of heat Killed Ed 1ct (BR's prep 4/13/02)

Tared tube empty
add 5mls
Cf 12000rpm 10"
Aspirate Sup
Re weigh tube = 0.0499 gm
Lyophize overnight re weigh

resuspended pellet in 2mls H2O
transfer to a pre-weighed 15 ml tube = 6.6018 gm
Freeze
Lyophilize
15 ml Tube Dry wt = 6.6064

Dry wt of pellet = .0046

Digest pLs88 pclpm3 + clone (#5) + Neg plasmid preps

w/ SalI + ClaI single digests

2ul DNA
1 ul Buffer
0.5ul Enzyme
6.5 ml H2O
10ml digest

ABI ~~Mastr~~ matrix stds came in. Stds are 4 colors: Blue, Red, Green, Yellow

mixed 1ul of each Standard with 12ul of deionized formamide (Borrowed from Hanson's Lab) 1 tube per std = 4 tubes 1 each color

Heat to 95C for 2"
Chill on ice

Sequence using Seq Run (250ul) E run module

injected each sample 3 times - this is done when the injection list is filled out

5

Date: - ~~Bt 5ml ...~~
- ~~Clone #5 5ml culture freeze~~
- ~~Weigh lyophilized heat killed Ed~~
- ~~Run digests on gel (in yellow box)~~
- ✓ prepare 2-750 ml cultures of heat Killed Ed. 1ct - Monday
- ✓ electroporate plasmid prep clone #5 into 11039 comp cells A11 D1-3 BHI strep plsdam2 Plate 100ul on 2 LB strep
- WT 93146 #19 pass from MW plate
- ~~Make 2 more 750ml BHI broth cultures~~
- ... gel stat

5/16/02 Thurs

Stds are still running on seq.

Weighed lypholized pellet of heat previous page

Run digests on gel pLr88 pclpn

Load entire Digest
6X dye is really conc. So only use

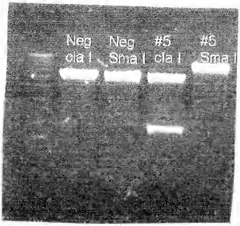
10ul DNA digested
1 ul 6X Dye
1 ul H2O

12ul on gel

0.7% ag

run 100 Volts

Lane 1 1ul 1Kb
2 Neg Cla I
3 Neg Sal I
4 #5 Cla I
5 #5 Sal I



Electroporate: plsdam2 into 11039 p.mult. electrocompetent cells

1ul plasmid prep #5 (yellow box) A8
40ul 11039 Comp cells box A11 D2

Tc = 5.12
2.5 KV
200 Ω
25 μF

Recovered 1 hr in 1ml LB 37C

plate 100ul neat on 2 LB Strep plates

Made 2 more 750ml broth cultures to grow 93146 WT #19 to heat ~~kill~~ Kill for vaccine trial

Signed [signature]

$\frac{37g}{l} \times \frac{750ml}{} = 27.75$ gms BHI

Read and Understood By

10

Notebook Number: _____

Date: _____

5/16/02 Thurs.

Froze

pls Dam 2 5vials Inl/each in LB glyc + 20% glycerol

Aquavac-Esc 5 vials Inl/each in BHI to 20% glycerol Vaccine

5/17/02 Fri:

Staff Appreciation Day.

Hotburg has grown yet on plates from Electroporation -

maybe need to be on BHI strep

Mutg 11039 Comp cells Dead

Leave at 37 C until Tomorrow

Passed 93146 WT #19 to new blood plate

Start 5 ml culture Sun

Took Rec + vaccine plates out of incubator Leave at RT

Start 5 ml BHI Cultures Sat.

5/19/02 - Started 5 ml BHI Cultures

5/20/02 MM leave Mark Sick BVR started 2 750ml cultures of WT #19

5/21/02 93146 WT Ed. ict take 1ml from each 750ml culture Combine in 1 tube

Streak for purity - plate is pure

plate 10^{-5} + 10^{-6} for colony Counts

10^{-5} TNTC TNTC

10^{-6} 286 287 Avg 286.5

Heat Kill at 60°C for 3 hours

plate for Viability - NO growth at 48 hrs

7/24/02

Blotted plates on Nitrocellulose membrane

Block in 0.01m PBS + 5% NFM 2 hrs RT

1° Ab used Straight Ed 9 1 hr RT

3X wash PBS 0.01M + Tw20

2° Ab goat α mouse Ig(H+L) AP 1/1500 4°C Overnight

Set up Serum Killing Assay see pg 17 for plate setup

Serum HI at 56°C for 30min

Read plate every 5min for 1 hr

plate 15 µl of 10^{-4} & 10^{-6} on BHI Ap

Colony Count results (7/25/02)

assay worked perfectly

E. coli	10^{-6}	10^{-7}	2.31×10^9
	27, 30, 47	0, 2, 3	1.56×10^9
7/26/02 WT	10^{-6}	10^{-7}	1.82×10^9
	35, 21, 26	2, 5, 9	3.56×10^9
RC	10^{-6}	10^{-7}	2.42×10^9
	44, 30, 35	0, 3, 6	2×10^9

3

Injected mice

100µl ip. of P. multocida WT 50, 100, 1000 2 mice per dose

" Dam 50, 100, 1000

" Dam 2 50, 100, 1000

Controls PBS 3 mice

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Signed

Signed

24 CO_2 indicates mouse was euthanized to relieve suffering

Notebook Number: _____

Date: _____

7/25/02 Mortality from mice Exp.

9:00am WT 100 2 mice 1st DOA 2nd CO_2 lethargic, shivering, flaccid, pic erection
WT 50 1 CO_2
Dam 100 1 DOA
Dam 100 1 DOA
Dam 2 100 1 DOA

Notes:
2nd mouse in WT 50 is very lethargic, does not respond to being handled
3rd mouse in Dam 100 is lethargic + shivering (due to fever?)

11:30am WT 100 CO_2 lethargic, Fever (shivering) does not respond to handling
Dam 100 1 DOA
Dam 50 1 DOA

Notes:
2nd mouse in Dam 50 is sick - shivering, non-responsive, pilo erection

1:30 No morts

4:45 WT 50 1 DOA
Dam 50 1 DOA
WT 100 1 DOA

All mice from Trt Groups WT 1000, WT 100, WT 50, Dam 1000 + Dam 50 are Dead

Dead mice were opined ventrally, the chest was spread open + they were stored in Sample cups w/ 10% buffered formalin

7/26/02

9.00am Dam 100 1 DOA

All mice from trt Group Dam 100 are Dead

7/27 - 7/28 Dan Scruggs ✓ mice

Mon 7/29/02

Start WT, R61 + E. coli Lux Cultures in ~~BHI~~ + 5ml Bilkup broth

Mice - Am all Trt groups are active + Alert
pm " " " "

8/28/02

Colony counts- Serum Killing assay E. coli Lux

10^{-7} 5
5
5
1.6×10^{10} CFU/mL

10^{-6} 35
51
85
3.7×10^{10} CFU/mL

8/29/02

R6 lux + WT Lux Colony Count plates were contaminated too badly to count

9/5/02 Inject mice

BVR prepared pmultocida 11039 WT + Dam2 for injections

1 xtra mouse uninjected

5 mice / dose 9 treatments

20 - 4 doses WT 5, 10, 50, 100

20 - 4 doses Dam 2 10, 50, 100, 500

5 control mice - PBS

Inject mice w/ 100ul each i.p.

use 1cc syringe with the 27 1/2 G needle

mice were not set up properly - use (Dan Scruggs + I) had to redistribute mice to 5/cage before injection — mice were stressed!

injected mice 11:30am ✓1:30 pm All OK ✓4:15pm all OK

Read and Understood By

Signed

Signed

Notebook Number: _____

Date: _____

9/6/02

√mice 8:00 am
WT 5 - 3 mortalities
1 lethargic, shivering, unresponsive
1 Almost dead completely unresponsive, unable to walk euthanize w/CO2
→ dead before I could euthanize
WT 10 - 4 morts
1 sluggish but responsive
WT 50 3 mort
2 sluggish but responsive
WT 100 3 mort
2 Sluggish but responsive have pilo erection
Dam2 500 All OK
100 all OK
50 all OK
10 all OK

PBS (-) control All OK

√mice 10:45 am

WT-100 both mice are shivering, lethargic, very rapid breathing

√mice 2:45 pm

WT 100 breathing very rapidly unresponsive
WT 50 " "
WT 10 Very lethargic
WT 5 Sick but alert

Dam2 - all alert but not as alert as controls

√mice 6:20 pm

WT No change
Dam2 No change
PBS No change

Notebook Number: Exhibit A Page 67

Date:

10-4-03

Actinobacillus - plasmid electroporation

1- Removed 40 µl of actinobacillus competent cells from microfuge tube and added to a cleam tube.
2- Added 1µl of plasmid DNA to the same tube.
3- Placed suspension on electroporation cuvette.
4- Set electroporator settings.
5- Electroporated and got a 5.1ms constant = 5.26.
6- Placed cells suspension in 1 mL BHI_{NAD}^{+}.
7- Incubated for 1 hour at 37°C.
8- Diluted cultures to 10^{-2}.
9- Plated 100 µl of 10^{0}, 10^{-1}, 10^{-2} on BHI_{NAD}^{+} strept plates.
10- Incubated plates at 37°C overnight.

Results

10-5-03

	Plate Count
10^{0}	33
10^{-1}	8
10^{-2}	0

Notebook Number:

Date:

10-29-02

Mutation Rates

1- Diluted XL1 Blue, XL1 dam-, Actin, and Actin dam- to 10^{-6}.
2- Plated in triplicate with 10 µL of 10^{5} and 10^{6}.
3- Plated 100 µL of overnight culture in triplicate on BHI,rif 100 µg/mL or BHINAD+,rif 100 µg/mL.
4- Incubated at 37°C overnight.

Results

Plate Counts	10^{-5}	10^{-6}
XL1 B	TNTC	TNTC
XL1 dam-	↓	↓
Actino	↓	↓
Actino dam-	↓	↓

BHI,rif

XL1	Bacterial lawn
XL1 dam-	Bacterial lawn
Actin	No growth
Actino dam-	No growth

Note: Will have to do antibiotic titer with Actinobacillus and rifampin

10.29.02

Fish Trail (24 hour)

1- Pooled 3 fish from each tank.
2- Removed spleen and kidney.
3- Weighed, macerated, diluted to 10^{-2}.
4- Plated 15 µL of 10^{0}, 10^{-1}, 10^{-2} on EIM plates.
5- Incubated at 26°C for 48 hours.

1- Started cultures of HB101 30.96 donor electroporation for plasmid preps.
2- Started cultures of 93146 WT, R6, and EC 11229 for MIC micro.

Killing with conjugation mutants

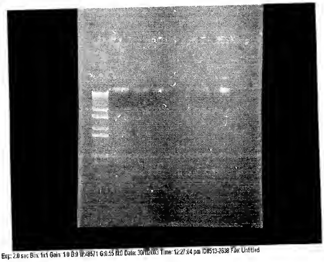
1- Pelleted bacteria in microtiter plate.
2- Resuspended in 100µl HBSS 0.9X with Ampicillin.
3- Removed 5µl from resuspended plate and added to 295µl HBSSamp.
4- Removed 5µl from the dilution plate and added to 35µl HBSSamp in white bottom assay plate.
5- Added an additional 40µl HBSS to control plate and 40µl normal serum to assay plate.
6- Incubated at RT for 1 hour.
7- Took readings on Lmax at Time 0, 30, and 60.
8- Analyzed data in Excel.
9- Highlighted mutants were frozen back (-80°C).

Results: See next page. (86)

1.30.03 HB101 30.96 donor plasmid prep.

1- Removed plasmid DNA with Qiagen kit.
2- Loaded 3µl on 7% agarose gel.
3- Ran at 100V for 1 hour.

Lane 1 - 1 Kb ladder
Lane 2 - prep 1
Lane 3 - prep 2
Lane 4 - prep 3
Lane 5 - prep 4
Lane 6 - prep 5
Lane 7 - prep 6



Exp: 2.0 sec Bin: 1x1 Gain: 1.0 B:0 W:48571 G:0.55 N:0 Date: 30/1/2003 Time: 12:27:04 pm ID#513-2638 File: Untitled

Signed

Signed